Cell, Vol. 119, 137–144, October 1, 2004, Copyright ©2004 by Cell Press

Resources

Combinatorial Marking of Cells and Organelles with Reconstituted Fluorescent Proteins

Shifang Zhang, Charles Ma, and Martin Chalfie*
Department of Biological Sciences
Columbia University
New York, New York 10027

Summary

Expression of GFP and other fluorescent proteins depends on *cis*-regulatory elements. Because these elements rarely direct expression to specific cell types, GFP production cannot always be sufficiently limited. Here we show that reconstitution of GFP, YFP, and CFP previously split into two polypeptides yields fluorescent products when coexpressed in *C. elegans*. Because this reconstitution involves two components, it can confirm cellular coexpression and identify cells expressing a previously uncharacterized promoter. By choosing promoters whose expression patterns overlap for a single cell type, we can produce animals with fluorescence only in those cells. Furthermore, when one partial GFP polypeptide is fused with a subcellularly localized protein or peptide, this restricted expression leads to the fluorescent marking of cellular components in a subset of cells.

Introduction

In the ten years since its introduction as a biological marker (Chalfie et al., 1994), green fluorescent protein (GFP) has become an important tool in many areas of biology (Chalfie and Kain, 2004). One advantage of GFP and similar fluorescent proteins is that they are genetically encoded and can be expressed in living cells and organisms from different promoters. This expression, however, is limited by the specificity of available promoters. Often cell specificity arises from the combinatorial action of multiple regulators, and individual cell types cannot be labeled using a single regulatory element. Here we show that these limitations can be overcome by the reconstitution of partial GFP polypeptides.

In the late 1990s, several investigators (Abedi et al., 1998; Baird et al., 1999; Doi and Yanagawa, 1999) demonstrated that the primary amino acid sequence of GFP could be interrupted at several positions by intervening coding sequences and still yield a fluorescent product. Ghosh et al. (2000) extended this research, showing that GFP, when split into two polypeptides (GFP[1–157] and GFP[158–238], which they named NGFP and CGFP, respectively), yielded a fluorescent product in vitro or when coexpressed in bacteria (Figure 1). This reconstitution only occurred, however, when these polypeptides were linked to sequences (NZ and CZ) that could form an antiparallel leucine zipper. They designated their constructs NZGFP (NGFP + 6 amino acid linker + NZ) and CZGFP (CZ + 4 amino acid linker + CGFP).

The Ghosh et al. results provided a proof of principle that production of fluorescence from partial GFP polypeptides, which we refer to as reconstituted GFP (recGFP), can be used to monitor protein-protein interactions. Hu et al. (2002) have subsequently used this property to demonstrate interactions among bZIP and Rel proteins in cultured cells. Nagai et al. (2001) developed another application involving the reconstitution of a fluorescent protein. They generated a calcium indicator by fusing partial yellow fluorescent protein (YFP) polypeptides (split at a different amino acid) to calmodulin and M13. The resulting fusion polypeptides reversibly produced fluorescence upon addition of calcium. These workers remarked, however, that the use of these peptides was compromised in HeLa cells because of competition by endogenous proteins.

Ozawa et al. (2000, 2001) used a slightly different approach to reconstitute GFP. They coupled two partial GFP polypeptides to calmodulin and to M13 but also included sequences for an intein, a domain for protein self-splicing, between these domains and the partial GFP polypeptides. Because of the inclusion of the intein, the two GFP polypeptides became covalently linked and severed from the other peptides when calcium mediated the interaction between calmodulin and M13 in *E. coli*. Umezawa (2003) reported the successful use of this intein-based method in cultured mammalian cells.

These examples show that recGFP can be used to demonstrate protein-protein interactions. In this paper, we describe other uses for recGFP. These additional uses stem from the fact that recGFP is a two-component system. A two-component system allows the direct demonstration of gene coexpression. Moreover, by using regulatory elements to drive overlapping, but not identical, patterns of gene expression, one can restrict fluorescence to cells that express both elements. Such restriction has several important applications.

Results

Reconstitution of Fluorescent Proteins In Vivo

We tested whether the Ghosh et al. NZGFP and CZGFP polypeptides could form fluorescent recGFP in eukaryotic cells by expressing them from the promoter for the *mec-18* gene ($P_{mec\text{-}18}$) in *C. elegans*. This promoter is only expressed in the six touch receptor neurons of this animal (G. Gu and M.C., unpublished data). Bright fluorescence was visible in these neurons when animals expressed both GFP-leucine zipper polypeptides from this promoter ($P_{mec\text{-}18}$*nzgfp* and $P_{mec\text{-}18}$*czgfp*; Figure 2A) but not when either NZGFP or CZGFP was expressed alone (data not shown). This fluorescence did not result from DNA rearrangement during *C. elegans* transformation because no fluorescence was seen in animals expressing $P_{mec\text{-}18}$*nzgfp* and *czgfp*, i.e., when CZGFP is not expressed from $P_{mec\text{-}18}$. Furthermore, the absence of CZ prevented the production of fluorescence (data not shown).

Production of recGFP was not promoter or tissue dependent, since it could be generated using the *hsp16.2*

*Correspondence: mc21@columbia.edu

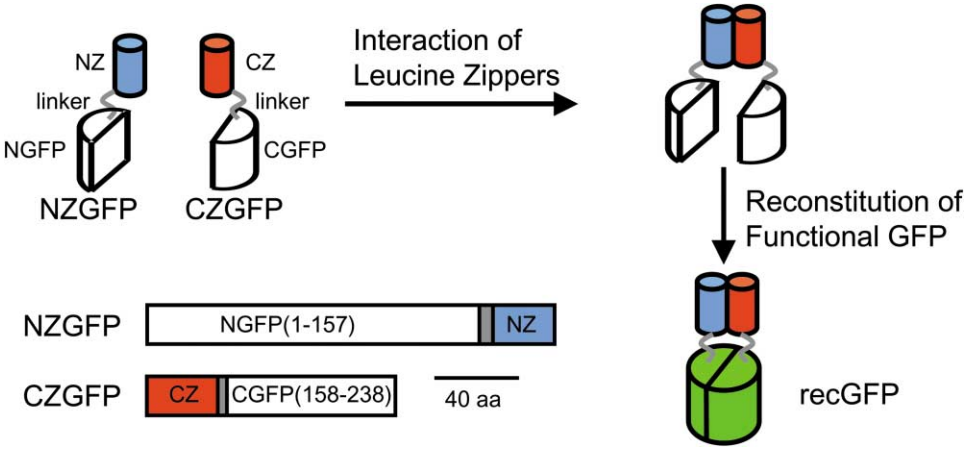

Figure 1. Schematic Representation of the Reconstitution of GFP through Antiparallel Leucine Zipper Domains

Each partial GFP-leucine zipper polypeptide (NZGFP and CZGFP) contains a leucine zipper domain (NZ, blue; CZ, red), a portion of GFP (NGFP, CGFP), and a linker (gray). Fluorescence (green) occurs when the NGFP and CGFP portions assemble together. The figure is based on the information in Ghosh et al. (2000).

heat shock promoter (Figure 2B), which is widely expressed, and the *unc-4* promoter, which is reported to be expressed in four types of motor neurons (SAB, VA, DA, and VC) (Lickteig et al., 2001; Miller and Niemeyer, 1995; Figures 2C and 2D).

The expression from the *unc-4* promoter revealed an unusual and potentially useful characteristic of the recGFP: it appeared to have a relatively shorter half-life compared to GFP. The *unc-4* gene is transiently expressed in different motor neurons at various times in *C. elegans* development. Because of the stability of GFP, this transient expression cannot be seen; young adult animals (2–3 days post hatching) contain fluorescent cells that have expressed GFP in the embryo, early larva, and late larva (Poyurovsky et al., 2003). In contrast, the only cells that fluoresce in young adults expressing a rapidly degraded GFP (caused by the fusion of the RING finger domain from the E3 ubiquitin ligase Mdm2) are the late larval cells (Poyurovsky et al., 2003). The animals with recGFP also displayed a similar loss in

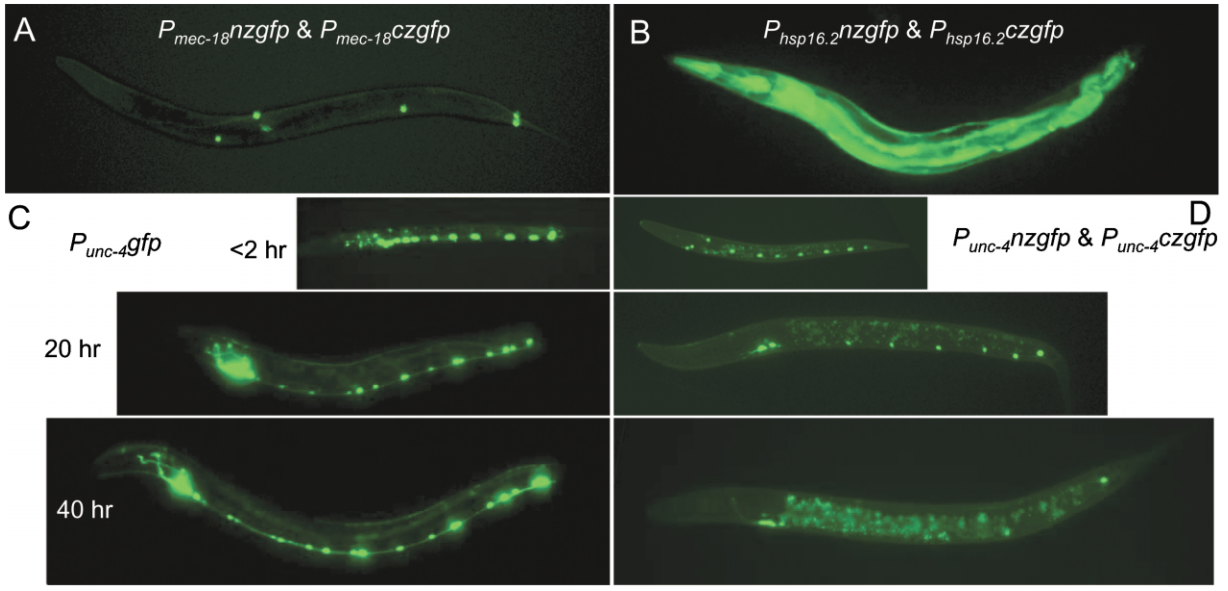

Figure 2. Expression of recGFP from Several *C. elegans* Promoters

(A) Expression of recGFP from the P_{mec-18} promoter in the six touch receptor neurons.

(B) Expression of recGFP from the heat shock promoter $P_{hsp16.2}$ throughout the animal.

(C and D) Comparison of fluorescence from GFP (C) and recGFP (D) in ventral cord neurons from the *unc-4* promoter at various times. Note that ventral cord fluorescence is missing in the 40 hr animal in (D) but not in (C). The numbers of fluorescent neurons in P_{unc-4}*gfp* animals were 6.9 ± 0.2 (mean ± SEM, n = 50 for all), 15.4 ± 0.3, and 17.0 ± 0.3 at <2, 20, and 40 hr after hatching, respectively. For animals with P_{unc-4}*nzgfp* and P_{unc-4}*czgfp*, the equivalent values are 6.4 ± 0.2, 5.4 ± 0.2, and 0.4 ± 0.1. The speckled fluorescence in the middle of some animals is due to intestinal autofluorescence.

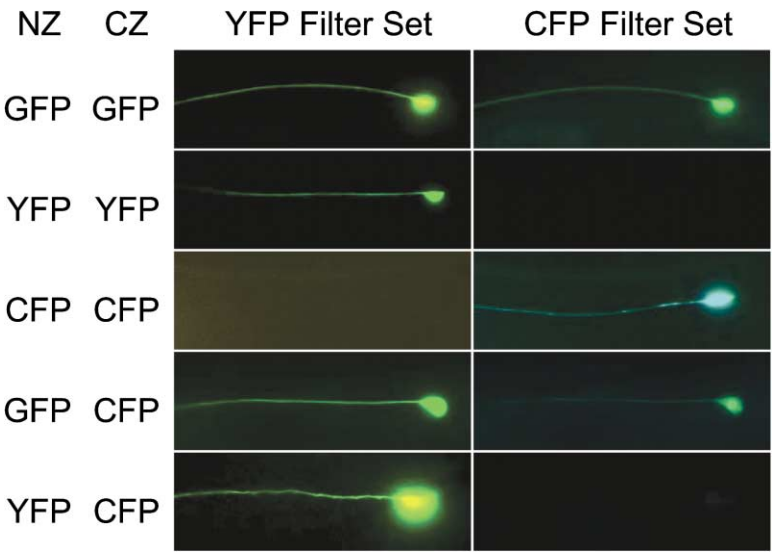

Figure 3. Reconstitution of Fluorescence using Split Fluorescent Proteins with Different Emission Spectra

The various CZ and NZ constructs are indicated to the left of the figure. All constructs were expressed from the *mec-18* promoter. Fluorescence using the YFP and CFP filter sets is shown. Images from both channels were processed identically. Some of the images appear cyan optically but green photographically when using the CFP filter set.

fluorescence as they matured (Figures 2C and 2D, particularly the last panel in each series which shows ventral cord fluorescence in adults in Figure 2C but not in Figure 2D).

As expected from the results of Nagai et al. (2001) and Hu and Kerppola (2003), reconstitution was not restricted to GFP (Figure 3). We obtained yellow-green and cyan fluorescence from recGFP variants of YFP and CFP. In addition, we found that CZCFP (i.e., CZGFP with the CFP mutation V163A) can be used generally with various forms of NZ fluorescent protein fusions. Fluorescence from recGFP was seen with both the Chroma YFP and CFP filter sets, whereas recYFP and recCFP were detected only with the appropriate filter set. The reconstituted fluorescent protein from NZGFP and CZCFP (recG/CFP) was detected with both filter sets (although stronger with the YFP filter set). In contrast, the reconstituted fluorescent protein from NZYFP and CZCFP (recY/CFP) was easily detected with the YFP filter set but hardly detectable with the CFP filter set. This last combination gave the most intense fluorescence of any of the combinations we tested (Figure 3). We also tested NZCFP and NZGFP with CZYFP, but they resulted in little or no fluorescence (data not shown).

Using recGFP to Demonstrate Gene Coexpression

To demonstrate that recGFP can identify cells that coexpress different promoters, we expressed NZGFP from the *unc-24* promoter and CZGFP from the *mec-2* promoter. The *unc-24* promoter is expressed in the *C. elegans* touch receptor neurons and in many cells in the ventral cord (T. Barnes and S. Hekimi, personal communication and Figure 4A); the *mec-2* promoter is expressed in the six touch receptor neurons (S.Z. et al., unpublished data). recGFP was formed only in the six touch receptor neurons (Figure 4B).

Because recGFP requires the combinatorial expression of two promoters (it acts as an "and" gate), it can overcome the limitation that GFP expression is dependent on available regulatory elements. To demonstrate the additional restriction possible with recGFP, we generated animals in which only the two FLP neurons fluoresced. No FLP-specific promoter has been reported, but *mec-3* and *egl-44*, genes that are expressed in several different cell types, are coexpressed only in these neurons (Way and Chalfie, 1989; Wu et al., 2001). By expressing NZGFP from the *mec-3* promoter and CZGFP from the *egl-44* promoter, we obtained animals with labeled FLP neurons (Figure 4C). (In some instances we observed animals with some touch neuron expression. We attribute this unexpected fluorescence to the production of recombined arrays in which the *mec-3* promoter drove both parts of recGFP. This unexpected expression was reduced when lower amounts of the construct with the *mec-3* promoter were used or when animals with each part of recGFP were made separately and then mated together. Two strains generated through such mating had only FLP fluorescence.)

Monitoring Changes in Gene Expression with recGFP

The ability of recGFP to visualize coexpression can also be used to demonstrate changes in gene expression. We tested this ability by examining the effects of mutations in the genes for the homeodomain transcription factor UNC-4 and the groucho-like transcription factor UNC-37 on the fate of motor neurons. Previously, Winnier et al. (1999) showed that mutations in *unc-4* and *unc-37*, which are expressed in and determine the fate of VA motor neurons, caused additional cells in the ventral cord to express the *acr-5* gene. We have confirmed this finding and demonstrated directly that the *unc-4*-expressing cells in the mutants expressed *acr-5* by showing that recGFP from $P_{unc\text{-}4}$*nzgfp* and $P_{acr\text{-}5}$*czgfp* formed in several ventral cord neurons in *unc-4* and *unc-37* mutants but not in wild-type (Figure 5); these cells are the VA motor neurons. We also found that several *unc-4*-expressing cells outside of the ventral cord (specifically, the SAB neurons and a cell we have tentatively identified as PDA) expressed *acr-5* even in wild-type animals. Interestingly, the intensity of fluorescence in these cells was brighter in the mutants than in

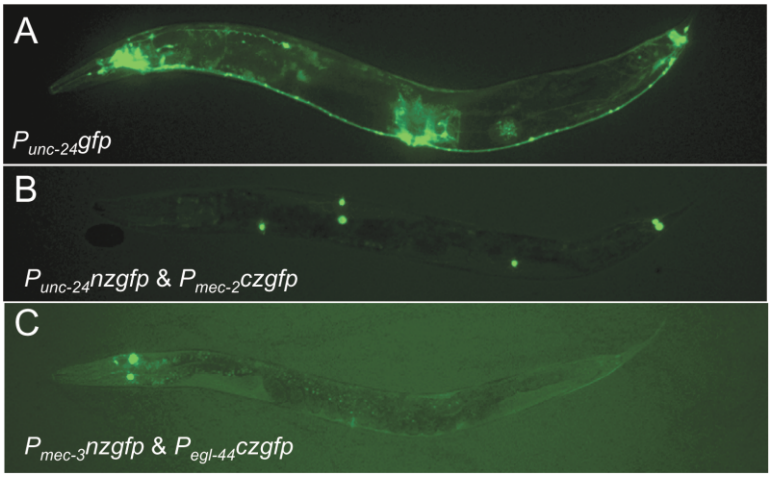

Figure 4. Use of recGFP to Identify Cells Coexpressing Two Genes

(A) $P_{unc-24}gfp$ is expressed in many adult cells. (B) $P_{unc-24}nzgfp$ and $P_{mec-2}czgfp$ are coexpressed only in six touch receptor neurons. (C) $P_{mec-3}nzgfp$ and $P_{egl-44}czgfp$ are coexpressed only in the two FLP neurons.

wild-type animals. Because *acr-5* is expressed in many cells, these observations could not have been easily made using coexpression of different color fluorescent proteins.

Using recGFP to Characterize Gene Expression

The combinatorial action of recGFP can also be used to identify cells expressing a particular gene. To demonstrate this property, we examined the expression of the *C. elegans sto-6* gene, a stomatin-encoding gene whose expression had been previously uncharacterized. $P_{sto-6}gfp$ is expressed in many of the motor neurons of the ventral cord (Figure 6A). To discover which neurons expressed *sto-6*, we used promoters that were known to be expressed in different classes of motor neurons in the ventral cord. We obtained recGFP fluorescence from $P_{sto-6}czgfp$ when NZGFP was generated from the *unc-4* and *acr-5* promoters, but not when it was generated from the *unc-47* promoter (Figures 6B–6D). These results indicate that *sto-6* is expressed in the ventral cord in the excitatory motor neurons (the VA, DA, and possibly VC neurons that express *unc-4* [Lickteig et al., 2001; Miller and Niemeyer, 1995] and the VB and DB motor neurons that express *acr-5* [Winnier et al., 1999]) but not the inhibitory motor neurons (the VD and DD motor neurons that express *unc-47* [McIntire et al., 1997]).

The apparent short half-life of recGFP raises an important caution about negative results in these experiments: promoters that are expressed at different times in the same cells may not produce a fluorescent product. For example, the HSN neurons in *C. elegans* express the *egl-44* gene in the embryo (Wu et al., 2001) and the *cat-1* gene, which is needed for the late larval expression of serotonin (Desai et al., 1988). HSN fluorescence was weak and rarely seen when recGFP was generated from these promoters (data not shown). Additionally, fewer cells than expected in adults fluoresced with $P_{sto-6}czgfp$ and $P_{unc-4}nzgfp$ (Figure 6B). Apparently, this expression was limited by the expression from the *unc-4* promoter. More cells were seen with this combination, however, than with $P_{unc-4}czgfp$ and $P_{unc-4}nzgfp$ (Figure 2D), presum-

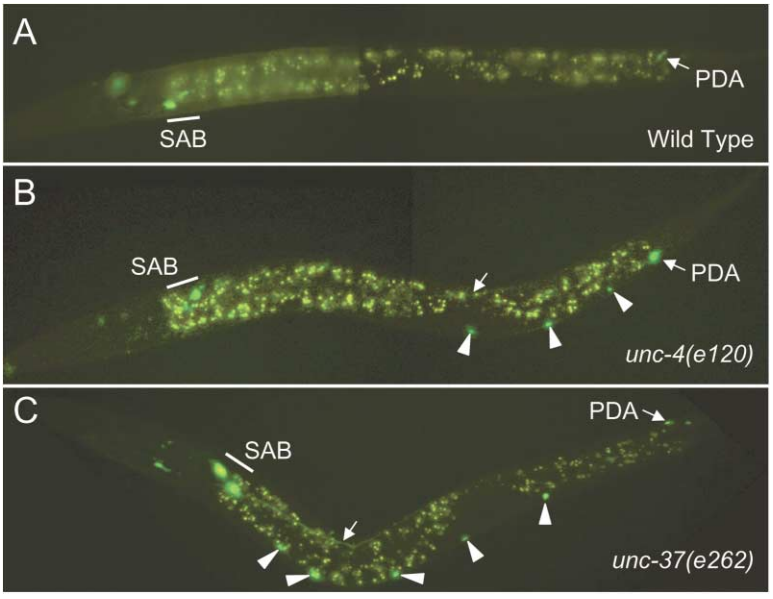

Figure 5. Use of recGFP Expressed from $P_{unc-4}nzgfp$ and $P_{acr-5}czgfp$ to Characterize Changes in Cell Fate

(A) In wild-type animals only the three SAB neurons (bar) and the PDA neuron (arrow) fluoresce; no fluorescence is seen in the ventral cord. (B) *unc-4(e120)* and (C) *unc-37(e262)* animals have fluorescent cells in the ventral cord (VA motor neurons, triangles). The more posterior of the SAB neurons (SABD, to the right in the figure) and the PDA cell are more intensely fluorescent in the mutant animals. The PDA process in the dorsal cord is seen in the mutants (unlabeled arrow) but not in wild-type. All animals are L2–L3 larvae.

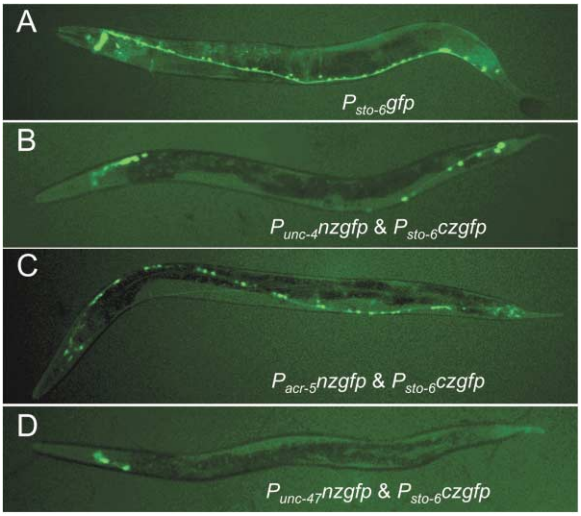

Figure 6. Use of recGFP to Characterize Gene Expression

(A) $P_{sto\text{-}6}$*gfp* is expressed in many cells in the head, ventral cord, and tail of an adult. Ventral cord fluorescence is found from (B) $P_{unc\text{-}4}$*nzgfp* and $P_{sto\text{-}6}$*czgfp*, (C) $P_{acr\text{-}5}$*nzgfp* and $P_{sto\text{-}6}$*czgfp*, but not (D) $P_{unc\text{-}47}$*nzgfp* and $P_{sto\text{-}6}$*czgfp* in adults.

ably because of the increased formation of the reconstituted protein due to mass action from the production of CZGFP from the *sto-6* promoter and possibly because of a greater stability of the reconstituted protein than of its parts. Although these results indicated that care should be used when expressing recGFP, they also indicate that these constructs can be used to study temporal as well as spatial coexpression.

Using recGFP to Restrict the Labeling of Cellular Components

The combinatorial action of recGFP can also be used to label cell constituents in a restricted set of cells. As an example, we have taken advantage of the finding that a synaptobrevin::GFP (SNB-1::GFP) protein fusion localizes to presynaptic vesicles (Nonet et al., 1998) to make a partial GFP version of this construct. SNB-1 was fused with CZGFP and expressed from the *sto-6* promoter. We also expressed NZGFP from the *acr-5* promoter. The resulting recGFP fluorescence localized in the B motor neurons of the ventral cord cells in puncta (the presynaptic regions) (Figures 7A and 7B). The addition of SNB-1 caused the localization of recGFP in the cells. Fluorescence localized to nuclei, however, when CZGFP had a 3× nuclear localization signal (Figure 7C).

Discussion

recGFP has the advantage over the use of two fluorescent proteins with distinct spectra or computational methods that allow separation of the fluorescence signals from multiple fluorescent proteins that it easily and unambiguously demonstrates coexpression. Specifically, the background fluorescence from either protein alone is eliminated, and the fluorescence from recGFP can be detected with standard fluorescence microscopy without the need for special equipment or programs.

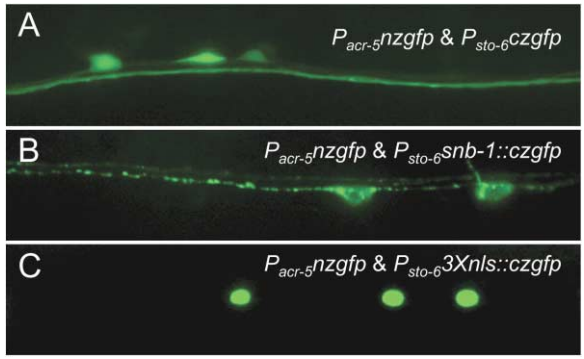

Figure 7. recGFP Can Be Used to Label Subcellular Components in Specific Sets of Cells

(A) $P_{acr\text{-}5}$*nzgfp* and $P_{sto\text{-}6}$*czgfp* label cell bodies and processes of the B motor neurons in ventral cord. Presynaptic regions (B) and nuclei (C) are labeled in these cells using $P_{acr\text{-}5}$*nzgfp* and $P_{sto\text{-}6}$*snb-1::czgfp* and $P_{sto\text{-}6}$*3Xnls::czgfp*, respectively.

The examples we provide in this paper demonstrating the usefulness of recGFP in vivo are only a subset of many potential uses for reconstituted fluorescent proteins. Intact GFP is currently used in a variety of ways in many organisms. In *C. elegans*, in addition to characterizing gene expression and protein localization, GFP has been used to label cells for electrophysiology (Goodman et al., 1998) and cell isolation (Zhang et al., 2002) and to generate organisms with labeled cells for subsequent genetic analysis (e.g., Du and Chalfie, 2001). The restricted expression (and apparent short half-life) of reconstituted fluorescent proteins should increase the usefulness of these and other methods. For example, reconstituted fluorescent proteins should allow more sensitive monitoring of changes in cell fate, differentiation, and function due to mutations, growth conditions, or exogeneously added reagents. The usefulness of reconstituted fluorescent proteins, however, should certainly not be restricted to *C. elegans*.

The combinatorial requirement for two partial fluorescent protein polypeptides means that recGFP and other fluorescent proteins should find application in several new uses. For example, recGFP could be used to demonstrate cell fusion, viral infection, and host-parasite interactions. Additionally, other partial fluorescent protein-derived polypeptides could be reconstituted using appropriate protein interaction domains. For example, the pH-sensitive GFP (pHluorin) of Miesenböck et al. (1998) and the photoactivatable GFP of Patterson and Lippincott-Schwartz (2002) should lend themselves to a similar splitting and reconstitution.

Finally, we suggest that recGFP could be used to more accurately describe gene expression patterns. Those of us who work with *C. elegans* take pride in the fact that all of the cells have been characterized (White et al., 1986). Unfortunately, many of the 302 *C. elegans* neurons are not easily identified. For example, few of the 163 neurons that crowd together in the head can be easily distinguished. As a result, many descriptions of gene expression patterns fail to identify all of the expressing cells. Although not unique to *C. elegans*, this inability to determine expression patterns accurately is,

perhaps, most frustrating with this animal because it is not based on lack of knowledge of the cell types; all the cells have been described. Reconstituted fluorescent proteins provide a means of correcting this problem, using our experiments deducing the expression pattern of the *sto-6* gene as a guide. An extension of these experiments would be the production of a set of tester strains in which NZGFP, NZYFP, and NZCFP (with or without nuclear localization signals) are expressed from well-characterized promoters. These strains could be mated with animals expressing CZCFP from a promoter whose expression was not known. With the color coding provided by the different NZ fluorescent proteins, relatively few (perhaps less than thirty) strains could be used to characterize gene expression in all of the 302 *C. elegans* neurons (118 classes) (White et al., 1986). Similar "identikits" could be constructed for *Drosophila*, zebrafish, mice, and other organisms.

Experimental Procedures

Nematode Maintenance

Animals were cultured at 20°C as described (Brenner, 1974) unless otherwise indicated. Wild-type (N2) and the *unc-4(e120)* and *unc-37(e262)* mutants have been described (Brenner, 1974).

Expression Constructs and Transformation

Bacterial expression plasmids pET11a-NZGFP and pET11a-CZGFP (Ghosh et al., 2000) for NZGFP and CZGFP, respectively, were gifts from Lynne Regan. The GFP sequence encoded by these plasmids differs from that of GFP listed as GenBank Accession Number P42212 in the following ways: F64L, S65C, Q80R (Chalfie et al., 1994), Y151P, I167T, and K238N. All of these changes were reported by Ghosh et al. (2000) except Y151P, which they reported as Y151L (in addition the coding sequence for CZ sequence in CZGFP begins MASA instead of MASE reported by these authors). The Y151P mutation and the E to A mutation in the CZ peptide occurred spontaneously during the cloning of the original pET11a expression vectors; the mutations did not affect the fluorescence phenotype in bacteria (T. Magliery and L. Regan, personal communication). The coding sequences of NZGFP and CZGFP were amplified by PCR with primers that introduced 5′ BamHI and 3′ EcoRI sites (these and the other primers used in this study are given in Supplemental Table S1 at http://www.cell.com/cgi/content/full/119/1/137/DC1/; the resulting plasmids are given in Table 1). The resulting PCR products were cut with BamHI and EcoRI and cloned into Fire promoterless GFP plasmid pPD95.77 (all the Fire vectors used in these studies are described at www.ciwemb.edu/pages/firelab.html). This procedure essentially replaced the original coding region of GFP in pPD95.77 with *nzgfp* or *czgfp*. pPD95.77 has artificial introns in the 5′ UTR, the GFP coding sequence, and the 3′ UTR that appear to stimulate GFP expression (S. Xu, G. Seydoux, and A. Fire, personal communication). All our constructs contain the 3′ UTR intron; addition of other introns to *nzgfp* and *czgfp* did not significantly improve recGFP fluorescence. For Figure 3, we used constructs with all of the Fire introns. The GFP sequence used for these constructs (from pPD95.77) has the S65C and Q80R mutations but none of the other changes found in the Ghosh et al. constructs.

We made partial YFP and CFP polypeptide-encoding plasmids by first replacing the GFP coding sequence in pPD95.77 with YFP coding sequence from pPD133.58 or CFP coding sequence from pPD133.51 using the fluorescent protein-coding AgeI–EagI fragment. We then made megaprimers (Brons-Poulsen et al., 1998) by amplifying the linker and zipper encoding regions of *nzgfp* and *czgfp* and used the Quikchange mutagenesis kit (Stratagene, La Jolla, California) to add them to pPD95.77. The primers were constructed so that amplification of pPD95.77 simultaneously deleted the unwanted fluorescent protein coding sequence and maintained the presence of all the artificial introns. These constructs produce YFP containing the same mutations (S65G, V68L, S72A, and T203Y) as 10C of Ormö et al. (1996) and CFP containing the mutations (Y66W, N146I, M153T, V163A) used by Miller et al. (1999). This CFP sequence is similar to W7 (Heim and Tsien, 1996), although this sequence lacks the N212K mutation. Protein-coding DNA sequences were verified (GeneWiz, Inc., North Brunswick, New Jersey).

Table 1. Plasmid List

Plasmid	Contents	Plasmid	Contents
TU#707	*nzgfp*	TU#722	P_{mec-2}*czgfp*
TU#708	*czgfp*	TU#723	P_{mec-3}*nzgfp*
TU#709	*ngfp*	TU#724	P_{unc-24}*gfp*
TU#710	*nzgfp*[a]	TU#725	P_{unc-24}*nzgfp*
TU#711	*czgfp*[a]	TU#726	$P_{hsp16.2}$*nzgfp*
TU#712	*nzyfp*[a]	TU#727	$P_{hsp16.2}$*czgfp*
TU#713	*czyfp*[a]	TU#728	P_{egl-44}*czgfp*
TU#714	*nzcfp*[a]	TU#729	P_{sto-6}*gfp*
TU#715	*czcfp*[a]	TU#730	P_{sto-6}*czgfp*
TU#716	P_{mec-18}*nzgfp*	TU#731	P_{sto-6}*snb-1::czgfp*
TU#717	P_{mec-18}*czgfp*	TU#732	P_{sto-6}*3Xnls::czgfp*
TU#718	P_{mec-18}*nzyfp*[a]	TU#733	P_{unc-4}*nzgfp*
TU#719	P_{mec-18}*czyfp*[a]	TU#734	P_{unc-4}*czgfp*
TU#720	P_{mec-18}*nzcfp*[a]	TU#735	P_{acr-5}*nzgfp*
TU#721	P_{mec-18}*czcfp*[a]	TU#736	P_{unc-47}*nzgfp*

All the plasmids were based on Fire vector pPD95.77, which contains a GFP-coding sequence with several artificial introns. Unless indicated, the derived vectors replace this sequence with a coding sequence without introns. Complete sequences of the plasmids are available from the authors.

[a] The GFP-coding sequences in these plasmids were derived from Fire vector pPD95.77 and have artificial introns.

We obtained the following promoter sequences (upstream sequences to the start codon) from genomic DNA or appropriate Fire (pPD) vectors using PCR primers that introduced the indicated restriction sites: *acr-5* (4.4 kb SphI-SphI fragment), *egl-44* (3.1 kb BamHI-BamHI fragment), *mec-2* (2.5 kb PstI-BamHI fragment), *mec-3* (1.9 kb PstI-BamHI fragment from pPD57.56), *mec-18* (0.4 kb HindIII-BamHI fragment), *hsp16.2* (0.4 kb SphI-BamHI fragment from pPD49.78), *sto-6* (2 kb SalI-BamHI fragment), *unc-4* (2.5 kb HindIII-BamHI fragment), *unc-24* (1.2 kb HindIII-BamHI fragment), *unc-47* (1.7 kb HindIII-BamHI fragment). In cases of nondirectional cloning, the correct orientation was verified by restriction digests. The entire genomic coding sequence of synaptic marker, *snb-1*, was amplified from pMN100.2 (a gift from Mike Nonet) and a BamHI site was added before its start codon and stop codon. This fragment was cloned into the P_{sto-6}*czgfp* construct at the BamHI site such that *snb-1* was downstream of the *sto-6* promoter and in-frame with *czgfp*. The orientation and sequence of the *snb-1* coding region were verified. The sequence containing three tandem repeats of the SV40 nuclear localization signal (*3Xnls*) was amplified from Fire vector pPD136.15 using primers that introduced 5′ BamHI and 3′ NheI sites. The amplified BamHI-NheI fragment was cloned into P_{sto-6}*czgfp* such that the *3Xnls* sequence was in-frame with the downstream *czgfp* sequence. The sequence of this localization signal was verified.

Transgenic animals were generated by microinjection using the pRF4 dominant roller plasmid (50 μg/ml) as a transformation marker (Mello et al., 1991). Expression plasmids were used at 50 μg/ml if injected alone or 25 μg/ml if two were injected. At least three stable lines were obtained for each genotype. All lines produced animals with similar fluorescence. When we examined recGFP expression from the *egl-44* and *mec-3* promoters, we used 5 μg/ml of the P_{mec-3}*nzgfp* and 45 μg/ml of the P_{egl-44}*czgfp* because higher concentrations of P_{mec-3}*nzgfp* resulted in occasional fluorescence in touch receptor neurons. When mating strains were generated, the one with P_{mec-3}*nzgfp* was made with the P_{ceh-22}*cfp* (Okkema and Fire, 1994) as an injection marker instead of pRF4.

Stability of recGFP

An integrated line carrying P_{unc-4}*gfp* was generated with γ ray irradiation. An integrated line carrying P_{unc-4}*nzgfp* and P_{unc-4}*czgfp* was gener-

ated by a spontaneous integration event. Both lines were maintained at 25°C. Animals were synchronized by collecting newly hatched larvae (within 2 hr) from plates from which larvae and adults had been removed with distilled water. The number of fluorescent ventral cord cell bodies was determined using epifluorescence at <2 hr (hatching), ~20 hr (L2/L3 larvae), and ~40 hr (L4 larvae/young adults).

Microscopy

Living L4 and young adult nematodes were viewed after being mounted on agarose pads (2% agarose, 50 mM Tris HCl, pH 8.5, 5 mM $MgCl_2$). For heat shocking, L4 or young adults were incubated at 32°C for 2 hr, transferred to 20°C, and viewed after approximately 12 hr. Animals were viewed by epifluorescence using a Zeiss Axioskop 2 microscope equipped with the following filter sets (Chroma Technology Corp., Rockingham, Vermont): (1) GFP: excitation D480/30×, dichroic 505DCLP, emission D605/55m; (2) YFP: excitation HQ500/20×, dichroic Q515LP, emission HQ520LP; (3) CFP: excitation D436/20×, dichroic 455DCLP, emission D480/40 m. Photographs were taken by a SPOT digital camera (Diagnostic Instruments, Inc., Sterling Heights, Michigan).

Acknowledgments

We thank Lynne Regan for the bacterial NZGFP and CZGFP plasmids, Josie Steinhauer for making the integrated $P_{unc\text{-}4}gfp$ strain, and Lisa Kole and members of our lab for helpful discussions. This work was supported by NIH grant GM30997.

Received: May 31, 2004
Revised: August 3, 2004
Accepted: August 5, 2004
Published: September 30, 2004

References

Abedi, M.R., Caponigro, G., and Kamb, A. (1998). Green fluorescent protein as a scaffold for intracellular presentation of peptides. Nucleic Acids Res. *26*, 623–630.

Baird, G.S., Zacharias, D.A., and Tsien, R.Y. (1999). Circular permutation and receptor insertion within green fluorescent proteins. Proc. Natl. Acad. Sci. USA *96*, 11241–11246.

Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. Genetics *77*, 71–94.

Brons-Poulsen, J., Petersen, N.E., Horder, M., and Kristiansen, K. (1998). An improved PCR-based method for site directed mutagenesis using megaprimers. Mol. Cell. Probes *12*, 345–348.

Chalfie, M., and Kain, S. eds. (2004). Green Fluorescent Protein: Properties, Applications and Protocols, 2nd ed. (New York: Wiley-Liss, Inc.), in press.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W.W., and Prasher, D.C. (1994). Green fluorescent protein as a marker for gene expression. Science *263*, 802–805.

Desai, C., Garriga, G., McIntire, S.L., and Horvitz, H.R. (1988). A genetic pathway for the development of the Caenorhabditis elegans HSN motor neurons. Nature *336*, 638–646.

Doi, N., and Yanagawa, H. (1999). Design of generic biosensors based on green fluorescent proteins with allosteric sites by directed evolution. FEBS Lett. *453*, 305–307.

Du, H., and Chalfie, M. (2001). Genes regulating touch cell development in *Caenorhabditis elegans*. Genetics *158*, 197–207.

Ghosh, I., Hamilton, A.D., and Regan, L. (2000). Antiparallel leucine zipper-directed protein reassembly: application to the green fluorescent protein. J. Am. Chem. Soc. *122*, 5658–5659.

Goodman, M.B., Hall, D.H., Avery, L., and Lockery, S.R. (1998). Active currents regulate sensitivity and dynamic range in *C. elegans* neurons. Neuron *20*, 763–772.

Heim, R., and Tsien, R.Y. (1996). Engineering green fluorescent protein for improved brightness, longer wavelengths and fluorescence resonance energy transfer. Curr. Biol. *6*, 178–182.

Hu, C.D., and Kerppola, T.K. (2003). Simultaneous visualization of multiple protein interactions in living cells using multicolor fluorescence complementation analysis. Nat. Biotechnol. *21*, 539–545.

Hu, C.D., Chinenov, Y., and Kerppola, T.K. (2002). Visualization of interactions among bZIP and Rel family proteins in living cells using bimolecular fluorescence complementation. Mol. Cell *9*, 789–798.

Lickteig, K.M., Duerr, J.S., Frisby, D.L., Hall, D.H., Rand, J.B., and Miller, D.M., 3rd. (2001). Regulation of neurotransmitter vesicles by the homeodomain protein UNC-4 and its transcriptional corepressor UNC-37/groucho in *Caenorhabditis elegans* cholinergic motor neurons. J. Neurosci. *21*, 2001–2014.

McIntire, S.L., Reimer, R.J., Schuske, K., Edwards, R.H., and Jorgensen, E.M. (1997). Identification and characterization of the vesicular GABA transporter. Nature *389*, 870–876.

Mello, C.C., Kramer, J.M., Stinchcomb, D., and Ambros, V. (1991). Efficient gene transfer in *C. elegans*: extrachromosomal maintenance and integration of transforming sequences. EMBO J. *10*, 3959–3970.

Miesenböck, G., De Angelis, D.A., and Rothman, J.E. (1998). Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. Nature *394*, 192–195.

Miller, D.M., 3rd, and Niemeyer, C.J. (1995). Expression of the *unc-4* homeoprotein in *Caenorhabditis elegans* motor neurons specifies presynaptic input. Development *121*, 2877–2886.

Miller, D.M., 3rd, Desai, N.S., Hardin, D.C., Piston, D.W., Patterson, G.H., Fleenor, J., Xu, S., and Fire, A. (1999). Two-color GFP expression system for *C. elegans*. Biotechniques *26*, 914–918, 920–911.

Nagai, T., Sawano, A., Park, E.S., and Miyawaki, A. (2001). Circularly permuted green fluorescent proteins engineered to sense Ca^{2+}. Proc. Natl. Acad. Sci. USA *98*, 3197–3202.

Nonet, M.L., Saifee, O., Zhao, H., Rand, J.B., and Wei, L. (1998). Synaptic transmission deficits in *Caenorhabditis elegans* synaptobrevin mutants. J. Neurosci. *18*, 70–80.

Okkema, P.G., and Fire, A. (1994). The *Caenorhabditis elegans* NK-2 class homeoprotein CEH-22 is involved in combinatorial activation of gene expression in pharyngeal muscle. Development *120*, 2175–2186.

Ormö, M., Cubitt, A.B., Kallio, K., Gross, L.A., Tsien, R.Y., and Remington, S.J. (1996). Crystal structure of the *Aequorea victoria* green fluorescent protein. Science *273*, 1392–1395.

Ozawa, T., Nogami, S., Sato, M., Ohya, Y., and Umezawa, Y. (2000). A fluorescent indicator for detecting protein-protein interactions in vivo based on protein splicing. Anal. Chem. *72*, 5151–5157.

Ozawa, T., Takeuchi, M., Kaihara, A.,Sato, M., and Umezawa, Y. (2001). Protein splicing-based reconstruction of split green fluorescent protein for monitoring protein-protein interactions in bacteria: improved sensitivity and reduced screening time. Anal. Chem. *73*, 5866–5874.

Patterson, G.H., and Lippincott-Schwartz, J. (2002). A photoactivatable GFP for selective photolabeling of proteins and cells. Science *297*, 1873–1877.

Poyurovsky, M.V., Jacq, X., Ma, C., Karni-Schmidt, O., Parker, P.J., Chalfie, M., Manley, J.L., and Prives, C. (2003). Nucleotide binding by the Mdm2 RING domain facilitates Arf-independent Mdm2 nucleolar localization. Mol. Cell *12*, 875–887.

Umezawa, Y. (2003). Seeing what was unseen: new analytical methods for molecular imaging. Chem. Rec. *3*, 22–28.

Way, J.C., and Chalfie, M. (1989). The *mec-3* gene of *Caenorhabditis elegans* requires its own product for maintained expression and is expressed in three neuronal cell types. Genes Dev. *3*, 1823–1833.

White, J.G., Southgate, E., Thompson, J.N., and Brenner, S. (1986). The structure of the nervous system of *C. elegans*. Philos. Trans. R. Soc. Lond. B Biol. Sci. *314*, 1–340.

Winnier, A.R., Meir, J.Y., Ross, J.M., Tavernarakis, N., Driscoll, M., Ishihara, T., Katsura, I., and Miller, D.M., 3rd. (1999). UNC-4/UNC-37-dependent repression of motor neuron-specific genes controls synaptic choice in *Caenorhabditis elegans*. Genes Dev. *13*, 2774–2786.

Wu, J., Duggan, A., and Chalfie, M. (2001). Inhibition of touch cell fate by *egl-44* and *egl-46* in *C. elegans*. Genes Dev. *15*, 789–802.

Zhang, Y., Ma, C., Delohery, T., Nasipak, B., Foat, B.C., Bounoutas, A., Bussemaker, H.J., Kim, S.K., and Chalfie, M. (2002). Identification of genes expressed in *C. elegans* touch receptor neurons. Nature *418*, 331–335.